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Studies on the Reduction and Re-formation of Protein Disulfide Bonds

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Previous communications have reported on the reduction of the disulfide bonds of ribonuclease (1–4), ribonuclease-S-protein (5), and lysozyme (6) by treatment of the native protein or protein derivative in 8 M urea solutions with thioglycolic acid or mercaptoethanol. Reduction by this procedure has a significant advantage over certain other techniques (*e.g.* borohydride (7) or sulfite reduction (8)) in that it yields reduced molecules that have suffered minimal, if any, covalent change other than that involved in the cleavage of the disulfide bonds to sulfhydryl groups. Thus, it has been observed that fully reduced ribonuclease contains no new NH_2-terminal amino acids, and that its molecular weight and amino acid content (after alkylation of the SH groups) are in accord with the expected values. It is therefore possible to study the re-formation of disulfide bonds and to examine the influence of chemical and environmental modifications on the efficiency of reoxidation and reactivation. It has been demonstrated that the eight sulfhydryl groups of fully reduced ribonuclease and of ribonuclease-S-protein undergo spontaneous oxidation to yield macromolecules that are extremely similar, and probably identical, to the parent molecule in their physical and enzymatic properties (4, 5).

The methods of reduction and subsequent manipulation that we have reported have, over the past year, been somewhat modified. We would like to summarize here some of the aspects of reduction, sulfhydryl group stabilization, and alkylation that appear to be of general use in the reduction of proteins, as well as some observations on conditions for regeneration of disulfide bonds in reduced ribonuclease.

EXPERIMENTAL PROCEDURE AND RESULTS

Reduction—In a typical experiment, 350 mg of native RNase (Sigma Chemical Company, lot R60-B-204, chromatographic grade) were dissolved in 10 ml of a freshly prepared 8 M solution of recrystallized urea, adjusted to pH 8.6 with 5% methylamine. Mercaptoethanol (Eastman-Kodak) was added at a level of 1 μl per mg of protein, the container was flushed with nitrogen, and the solution was allowed to stand for 4½ hours at room temperature. Methylamine was employed for neutralization to insure the decomposition of any thioglycollides (3) that might be present in the reducing agent. After this period, the pH was adjusted to 3.5 with glacial acetic acid and the entire solution was applied to a 2.5- × 35-cm column of crossed-linked dextran (9) (Sephadex G-25, Pharmacia Company, Uppsala, Sweden) which had been thoroughly equilibrated with 0.1 M acetic acid. The column was developed with the same solvent. As shown in Fig. 1, the bulk of the protein emerged considerably ahead of the reagents in the original mixture. (In experiments using longer columns or smaller applied volumes of reaction mixture, the mercaptoethanol, urea, and salts appear in a fraction entirely separated from the protein peak by approximately 20 to 25 ml of effluent volume; see, for example, Fig. 2.) Titrations with PCMB[1] (10) and estimations of SH content by radioactivity determinations of C^{14}-iodoacetate-treated material (5) from two portions of the major part of the peak gave the same values (see legend to Fig. 1). In samples from the trailing portion of the peak, determinations with PCMB gave high results because of slight contamination with mercaptoethanol. Values obtained by the radioactive method on these latter samples were not so affected, since the derivatives were submitted, after radioactive alkylation, to a second passage through Sephadex G-25 before comparison of their specific radioactivities with that of the C^{14}-iodoacetate reagent.

The method of reduction and purification described above has also been applied to trypsinogen, chymotrypsinogen, and lysozyme. Fully reduced products that were soluble in the dilute acetic acid solvent were obtained from each of these proteins.

Reoxidation—A reinvestigation of the methods used for the oxidation of reduced RNase was undertaken in an attempt to increase the yields of regenerated native enzyme and, particularly, to decrease the production of insoluble side products. High yields of enzymatically active material and the absence of precipitation are achieved only when oxidation is carried out at low protein concentrations (Table I) and when conditions of surface denaturation (*e.g.* bubbling with air) are avoided. As shown in Table I, efficient oxidation occurs when dilute solutions, adjusted to pH 8.0 to 8.5, are allowed simply to stand in open vessels at room temperature for approximately 20 hours. Under these conditions, the yields of regenerated, active enzyme have been uniformly between 80 and 100%.

Conversion of SH groups in reduced RNase to disulfide bonds is extremely slow at the hydrogen ion concentration of the acetic acid solution employed for the Sephadex columns. At room temperature and pH 3.0, a sample of protein containing 7.8 SH groups per mole contained, after 24, 72, and 96 hours of exposure to atmospheric oxygen, respectively, 7.5, 6.9, and 4.5 SH groups per mole. At icebox temperatures, the SH group content is undiminished for 2 to 3 days.

Stabilization of Protein SH Groups after Reduction—In studies of the sulfhydryl-disulfide relationships in proteins, SH groups are generally stabilized by reaction with suitable alkylating re-

[1] The abbreviation used is: PCMB, *p*-chloromercuribenzoate.

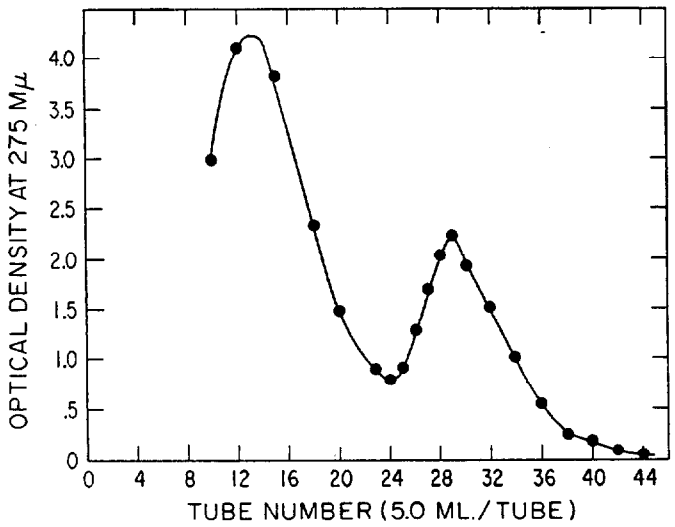

FIG. 1. The separation of reduced ribonuclease from reagents. After reduction, the solution of reduced protein was adjusted to pH 3.5 with glacial acetic acid and applied, in a volume of 12 ml, to a 2.5- × 25-cm column of Sephadex G-25 which had previously been equilibrated with 0.1 M acetic acid. Samples were taken from tubes 12, 15, 18, and 20 for estimation of SH content by titration with PCMB (10) and by determination of radioactivity after alkylation with iodoacetic acid-1-C^{14} (5) (see the text). The latter method yielded 7.5, 7.4, 7.7, and 7.7 moles of SH per mole of RNase whereas the PCMB titrations gave 7.6, 7.7, 11.5, and 20 moles of SH per mole of protein for the same samples. This difference in the results obtained by the two methods indicates the presence, in the two tubes from the trailing portion of the protein peak, of mercaptoethanol that was not fully resolved from the reduced protein on this particular column.

TABLE I

Oxidation of reduced RNase at various protein concentrations

The reduced protein was prepared as described in the text and freed from reagents on Sephadex G-25. The protein in the column effluent, after appropriate dilution, was adjusted to pH 8.5 and allowed to stand without stirring at room temperature for 20 hours, at which time the proportion of soluble protein and the enzyme activity of this fraction were estimated.

Concentration of reduced protein	% Yield of soluble protein	Activity of soluble fraction; % of equivalent concentration of native RNase	% Regeneration of activity
mg/ml			
7.0	27	31	8
4.8	42	70	29
2.3	87	75	65
0.9	100	77	77
0.35	100	94	94

agents such as iodoacetic acid, iodoacetamide, or *N*-ethylmaleimide. In spite of care in the maintenance of proper pH values and times of reaction, experience in various laboratories has indicated that alkylation of groups other than SH groups may occur in small but significant amounts (11) (*e.g.* methionine sulfur to the sulfonium derivative, lysine ε-amino groups to the *N*-alkyl derivative). For this reason we have recently employed an excess of mercaptoethanol to immobilize the iodoacetate at the end of the alkylating period.

The reduction mixture is diluted before alkylation with 0.5 M Tris-acetate buffer, pH 8.5, to reduce the urea concentration to approximately 2 M. At this level of urea, nonspecific alkylations are reduced to a level of 0.1 to 0.2 mole of alkyl groups per mole of protein, as estimated with C^{14}-iodoacetate (5). The diluted, reduced protein is then treated with a 10-fold excess of iodoacetic acid (calculated on the basis of the mercaptoethanol present in the reduction mixture) at room temperature for 12 to 15 minutes. The presence of buffer obviates the necessity of constant monitoring of pH during the alkylation. At the end of the reaction, a 10-fold excess of mercaptoethanol is added (calculated on the basis of the added iodoacetic acid). The mixture is then allowed to stand for 30 to 60 minutes to permit complete reaction of free iodoacetate and is finally passed through Sephadex G-25 to yield the reagent-free, salt-free protein derivative. This material is now ready for radioactive counting for the determination of *S*-carboxymethyl group content. Counting is most conveniently done in a liquid scintillation counter, the protein solution being mixed directly with a water-miscible phosphor solution (12). Specific radioactivities have generally been calculated on the basis of protein concentrations estimated by spectrophotometric determinations.

To stabilize the SH groups in a *reversible* form, the reduced protein emerging from the Sephadex column is adjusted to approximately 5 mg per ml with 0.1 M acetic acid and treated with a 1.1-fold excess of sodium PCMB (based on the moles of SH groups), dissolved in water, and adjusted to pH 8. Although PCMB is quite insoluble in 0.1 M acetic acid, the added material reacts rapidly with the free SH groups of the protein derivative,

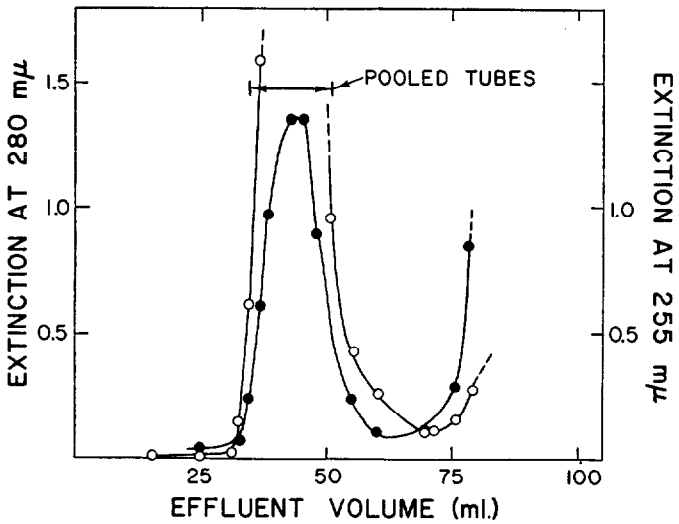

FIG. 2. Regeneration of reduced RNase from its compound with PCMB. The PCMB compound (110 mg) was dissolved in 10 ml of 0.1 M acetic acid by the addition of 100 μl of mercaptoethanol. After standing for 30 minutes, the solution was passed through a 2.5- × 30-cm column of Sephadex G-25 previously equilibrated with 0.1 M acetic acid. The column was developed with the same solvent, and fractions of 2.5 ml each were collected. The odor of mercaptoethanol was first detected in the vicinity of 65 ml effluent volume. The tubes indicated in the figure were pooled and the content of SH groups per mole of RNase was determined by titration with PCMB (10). The value obtained (7.4 SH groups per mole) agrees well with that found for the original reduced RNase (7.5 SH groups per mole) which served as starting material for the preparation of the PCMB derivative. The recovery of absorbancy units (measured at 280 mμ in the pooled tubes) was 65%. Absorbancy was measured at 280 mμ (○) to detect the reduced protein, and at 255 mμ (●) to detect the emergence of mercaptoethanol and its PCMB derivative.

and marked precipitation is first observed only when the point of equivalence has been reached. The final mixture may be dialyzed against 0.1 M phosphate buffer at pH 8 to remove excess PCMB and, after further dialysis against water, the suspension of PCMB-RNase is lyophilized for storage. The derivative (reduced RNase-$8C_7H_5O_2Hg$; calculated Hg content, 9.9%; found 9.1% (13), 10.4% (14)) is somewhat soluble in 0.1 M phosphate buffer, pH 8.0 (approximately 0.5 mg per ml), and very soluble in strong urea solutions. It may be stored in the refrigerator for at least 2 months without detectable loss in its ability to regenerate fully reduced RNase after removal of the PCMB groups. Such removal is accomplished by suspending the derivative in 0.1 M acetic acid (10 to 20 mg per ml), adding 1 μl of mercaptoethanol per mg of derivative (whereupon the solution becomes clear), and passing the solution through the Sephadex column as described above (Fig. 2). The resulting reagent-free protein contains the sulfhydryl group content of the original reduced material and may be converted to active enzyme in high yield by oxidation.

The availability of the PCMB derivative of reduced RNase and its ready oxidation to active enzyme after removal of the masking groups make possible a study of the ability of the reduced chain to re-form enzymatically active material after modification by limited selective cleavage with proteolytic enzymes. Experiments with the Jacobsem-Leonis autotitrator (15) (pH-Stat) indicate that the PCMB derivative of reduced RNase is digested with trypsin to an extent equivalent to that of reduced carboxymethylated material (6). Thus, the number of peptide bonds cleaved is that expected from the lysine and arginine content of the chain (*i.e.* 12 bonds cleaved per mole, with two bonds unreactive, the NH_2-terminal lysyl bond and the lysyl bond in the sequence, Lys.Pro).

DISCUSSION

The main point to be emphasized in connection with the methods described above is the relatively gentle nature of the procedures for reduction and for subsequent separation on columns of the reduced protein derivative from reagents. The high yield of active regenerated RNase (Table I) obtained when oxidations of SH groups are carried out at low concentrations of reduced protein is in strong contrast to the rather poor yields obtained after reduction by a reagent such as sodium borohydride (16) which is known to cause limited, but probably highly critical cleavage of peptide bonds (17). Perhaps the best evidence for the mildness of the methods comes from experiments, to be described in detail in a separate communication, in which RNase was subjected to two successive rounds of reduction and air oxidation without significant loss of activity.

The stability of the bulk of the covalent structure of RNase to conditions of reduction, combined with the ability of the reduced molecule to undergo spontaneous oxidation and re-formation of secondary and tertiary structure, permits an approach to the preparation of fragments of the RNase chain that might recombine, through disulfide bonds, to yield enzymatically active compounds.

SUMMARY

Methods are described for the reduction of disulfide bonds in ribonuclease and other proteins, for the separation of the reduced derivatives on columns of cross-linked dextran (Sephadex), and for the convenient carboxymethylation of SH groups produced. The reductive technique employed does not appear to alter other covalent features of ribonuclease, as evidenced by the oxidizability of the reduced chain to yield a product with native enzymatic and physical characteristics. Conditions are described for optimal regeneration of activity by exposure of dilute solutions to molecular oxygen. The preparation of a *p*-mercuribenzoic acid derivative of the reduced protein is described. This material serves as a stable form of the fully reduced chain for degradation with proteolytic enzymes.

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